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# Collaborative Complementation Study of Temperature-Sensitive Mutants of Herpes Simplex Virus Types 1 and 2

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Twenty-three complementation groups of herpes simplex virus type 1 (HSV-1) and 20 of HSV-2 were identified by qualitative and quantitative complementation analysis from among 43 temperature-sensitive (*ts*) mutants of HSV-1 and 29 *ts* mutants of HSV-2 which had been isolated independently in 10 laboratories.

The herpes simplex virus (HSV) genome is large ($100 \times 10^6$) and complex (5, 25, 31) and probably encodes from 70 to 80 genes. A complete understanding of the structural and functional organization of the genome will necessitate the identification of all viral genes and their ordering on the viral genetic map.

To date, most genetic studies of HSV have utilized temperature-sensitive (*ts*) conditional lethal mutants which can occur, theoretically, in all essential genes. Although the total number of essential genes encoded by HSV DNA is not known, complementation analysis of one series of *ts* mutants has led to the identification of 15 cistrons of HSV type 1 (HSV-1) (22) and, in a collaborative study, 18 cistrons of HSV-2 (30). Because it is very likely that more than this number of essential cistrons is encoded by the HSV genome (3), a collaborative complementation study was undertaken to identify additional essential cistrons from among existing series of HSV-1 and HSV-2 *ts* mutants.

The results of this study establish the identity of 23 cistrons among 43 *ts* mutants of HSV-1 and 20 cistrons among 29 *ts* mutants of HSV-2 and demonstrate that most previously untested mutants belong to already established complementation groups. It is clear that the number of new complementation groups identified in this study is smaller than one would expect given the number of independently derived mutants tested. These findings demonstrate the necessity, therefore, for (i) developing new procedures for the isolation of mutants in as yet unidentified HSV cistrons and (ii) examining the functional basis for the extensive failure to complement observed with certain mutants.

## MATERIALS AND METHODS

**Cells and cell culture.** Monolayer cultures of serially propagated human embryonic lung (HEL) fibroblasts were used for the preparation of virus stocks and for mixed infections in the quantitative complementation test. Vero cell monolayers were used for virus assay and for mixed infections in the qualitative complementation test. Both HEL and Vero cells were grown at 37°C in Eagle medium (Autopow, Flow Laboratories, Rockville, Md.) supplemented with 10% fetal bovine serum and containing 0.075% $NaHCO_3$ (for cultures in closed vessels) or 0.225% $NaHCO_3$ (for cultures in open vessels in a 5% $CO_2$ atmosphere).

**Viruses and virus assays.** Properties of the seven series of HSV-1 and five series of HSV-2 *ts* mutants used in this study are shown in Table 1. Wild-type virus strains and the mutagens used for the induction of *ts* mutants, as well as the viral DNA phenotypes of the mutants, are shown. Each mutant from a given series included in the study had been shown previously to represent an unique complementation group in that series. Only one member of each group was tested.

Virus assays were performed in Vero cells by using a 2% methylcellulose overlay (7) in $CO_2$ incubators (Wedco, Silver Spring, Md.) with temperature variations of ±0.2°C.

**Preparation of virus stocks.** Stocks of *ts* mutants were prepared by infecting confluent HEL cell monolayers in 100-mm petri plates with 1 ml of virus inoculum at multiplicities of 0.01 to 0.1 PFU/cell. After adsorption at 37°C for 1 h, the inoculum was decanted, monolayers were washed once with Tris buffer at pH

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TABLE 1. *Selected properties of the HSV-1 and HSV-2 ts mutants used in this study*

| Virus type | Wild-type strain | Mutagen[a] | Mutant | Viral DNA phenotype[b] | Reference or investigator |
|---|---|---|---|---|---|
| HSV-1 | KOS | BUdR | A1 | − | 1, 21 |
| | | BUdR | B2 | − | |
| | | BUdR | C4 | − | |
| | | BUdR | D9 | − | |
| | | BUdR | E6 | + | |
| | | NTG | F18 | + | |
| | | BUdR | G3 | ± | |
| | | BUdR | I11 | + | |
| | | NTG | J12 | + | |
| | | NTG | K13 | ± | |
| | | NTG | L14 | ± | |
| | | UV | M19 | ± | |
| | | UV | N20 | + | |
| | | UV | O22 | ± | |
| | | AP | P23 | − | J. Jofre, C.-T. Chu, and P. A. Schaffer, manuscript in preparation |
| | 17 | BUdR | A | + | 3 |
| | | BUdR | D | − | |
| | | BUdR | F | + | |
| | | BUdR | G | + | |
| | | BUdR | I | + | |
| | KOS 1.1 | BUdR | 84 | −[c] | 14 |
| | | BUdR | 478 | + | |
| | | BUdR | 656 | − | |
| | | BUdR | 661 | − | |
| | | BUdR | 901 | − | |
| | | BUdR | 822 | + | |
| | | BUdR | 833 | − | |
| | HFEM | BUdR | B1 | − | A. Buchan |
| | | BUdR | B5 | + | |
| | | BUdR | B7 | − | |
| | | NA | N103 | − | |
| | HFEM | Spontaneous | LS1 | + | 11 |
| | | BUdR | LB1 | + | |
| | | BUdR | LB2 | ± | |
| | | Spontaneous | LS2 | + | |
| | | BUdR | LB3 | + | |
| | | BUdR | LB4 | + | |
| | | BUdR | LB5 | + | |
| | KOSpp601 | BUdR | 3 | ND[d] | V. Vonka |
| | | BUdR | 7 | ND | |
| | 13 | BUdR | D10 | − | 17 |
| | | BUdR | C4 | + | |
| | | BUdR | G5 | + | |
| HSV-2 | 186 | BUdR | A8 | − | 9, 20 |
| | | BUdR | B5 | − | |
| | | BUdR | C2 | ± | |
| | | BUdR | D6 | + | |
| | | BUdR | E7 | + | |
| | | BUdR | F3 | + | |
| | | BUdR | G4 | + | |
| | | UV | H9 | − | 4 |

TABLE 1—*Continued*

| Virus type | Wild-type strain | Mutagen[a] | Mutant | Viral DNA phenotype[b] | Reference or investigator |
|---|---|---|---|---|---|
| | HSG52 | BUdR | 1 | − | 12, 13, 29 |
| | | BUdR | 2 | − | |
| | | BUdR | 3 | + | |
| | | BUdR | 4 | + | |
| | | BUdR | 5 | + | |
| | | BUdR | 8 | − | |
| | | BUdR | 9 | − | |
| | | BUdR | 11 | − | |
| | | BUdR | 12 | ± | |
| | | BUdR | 13 | ± | |
| | IPB2 | NA | 1 | ND | 33 |
| | | NA | 42082 | ND | |
| | 333 | UV | 69 | + | 15, 32 |
| | | BUdR | 74 | ± | |
| | UW268 | BUdR | 1 | + | 28 |
| | | BUdR | 5 | + | |
| | | BUdR | 6 | − | |
| | | BUdR | 11 | − | |
| | | BUdR | 12 | + | |
| | | BUdR | 19 | − | |
| | | BUdR | 33 | + | |

[a] BUdR, 5-Bromodeoxyuridine; NTG, nitrosoguanidine; AP, 2-aminopurine; NA, nitrous acid.

[b] The viral DNA phenotype of *ts* mutants is based upon their ability to synthesize viral DNA at the nonpermissive temperature compared with the ability of the wild-type virus to synthesize viral DNA at this temperature as 100%. +, >20%; ±, ≤20%; −, no detectable viral DNA synthesized.

[c] Viral DNA phenotypes of strain KOS 1.1 *ts* mutants were kindly supplied by Myron Levine, University of Michigan, Ann Arbor, Mich.

[d] ND, Not done.

7.4, and 7 ml of Eagle medium containing 5% fetal bovine serum and 0.225% $NaHCO_3$ was added to each plate. Infected cells were incubated at 34°C until cytopathic effects were generalized. Cells were then scraped into the medium, and replicate suspensions were pooled. The suspension was sonically disrupted for 45 s at 10 kc and centrifuged at 180 × *g*, 5°C for 10 min. The supernatant fluid was dispensed, frozen at −90°C, and assayed at permissive (34°C) and nonpermissive (38°C for HSV-2 and 39°C for HSV-1 *ts* mutants) temperatures. Virus preparations containing significant levels of $ts^+$ revertants were plaque-purified, and stocks were prepared from revertant-free plaques. All virus stocks used in this study exhibited efficiencies of plating [EOP; (PFU per milliliter, assayed at nonpermissive temperature)/(PFU per milliliter, assayed at permissive temperature)] of less than or equal to $10^{-4}$. Mutants with consistently high levels of leak and reversion (efficiency of plating > $10^{-4}$) were not included in these studies.

**Complementation tests.** Two types of complementation tests were employed in this study. The qualitative test (4) was used for initial screening purposes, and the quantitative test (22) was used as the more definitive test. These two tests differ primarily in the time permitted for the exchange of gene products and for virus replication during mixed infection. The quantitative test involves a single round of virus replication, whereas the qualitative test involves multiple rounds of replication. Correlation of results obtained in the two types of tests has generally been very good; however, false negative and false positive qualitative results have been described (4, 30).

**(i) Qualitative complementation tests.** Qualitative tests were conducted as described previously (4). Briefly, Vero cells were infected with two mutants by placing filter paper disks saturated with mixtures containing $2 \times 10^6$, $4 \times 10^5$ and $8 \times 10^4$ PFU of each mutant per ml on preformed monolayers in 60-mm petri dishes. Control disks were saturated with suspensions of individual viruses at these dilutions. After incubation at the nonpermissive temperature for 5 days under 2% methylcellulose, neutral red was added to each plate. Clearing of the monolayers in the areas of mixed infections was compared with the clearing produced in areas inoculated with each mutant alone. If more marked clearing occurred in areas of mixed infections than in areas infected with each mutant alone, complementation was said to have occurred.

**(ii) Quantitative complementation tests.** The quantitative or yield-of-progeny virus test compares the yield of virus obtained from cells infected with two

*ts* mutants and incubated at the nonpermissive temperature with the yield obtained from cells infected with each mutant alone and incubated at this temperature. The test was carried out as previously described (22). Tube cultures of HEL cells were inoculated at a multiplicity of 2.5 PFU of each virus per cell (a combined multiplicity of 5), and singly infected control cultures were inoculated with 5 PFU of each virus alone per cell. Infected cultures were incubated for 18 h at the nonpermissive temperature in water baths (Blue M, Rock Island, Ill.) with temperature variations of ±0.1°C, and virus yields were titrated in Vero cell monolayers. Complementation indices (CI) were calculated from the formula: $CI = ([A + B]_{39°C})/([A]_{39°C} + [B]_{39°C})$, where A and B are two mutants, and infected cells were incubated at the nonpermissive temperature. Virus yields were assayed at the permissive temperature. As in previous tests (22), a value of 2 or greater was taken to indicate positive complementation.

In the present study each mutant pair was tested by both procedures. If the qualitative test was clearly positive and the quantitative test yielded a CI of 10 or greater, no additional tests were performed. If one type of test indicated complementation and the other did not, both tests were repeated at least once. Unless otherwise stated, the result of the quantitative test was considered to be definitive. Quantitative tests of mutant pairs which failed to complement in initial tests were repeated three times.

## RESULTS

**Quantitative complementation tests with *ts* mutants of HSV-1. (i) Establishment of the standard set of HSV-1 *ts* mutants.** Rather than undertaking the analysis of all HSV-1 *ts* mutants together, complementation tests were first performed between mutants derived from strains KOS and 17 to obtain a standard set of mutants with which to compare mutants isolated independently from other strains. Five *ts* mutants of strain 17 and 15 *ts* mutants of strain KOS were tested in all possible pairwise combinations. The results of these tests are presented in Table 2. Mutants *ts*D, F, G, and I of strain 17 failed to complement mutants in at least one of the strain KOS complementation groups. Mutants *ts*J12 and L14 of strain KOS and mutants *ts*D and F of strain 17 failed to complement mutants in two different complementation groups. These data suggest that two or more of these mutants are double mutants or that the complementation between strain 17 *ts* mutants F and I and D and F is intracistronic. The factor which argues most strongly against intracistronic complementation is that if this were the case, F, I, and D (or three of five mutants of strain 17) would have to contain mutations in the same gene. This situation would require extremely strong selectional artifact and is, therefore, unlikely. More plausible is the possibility that *ts*J12 and *ts*L14 are double mutants. Characteristic of a point mutation, the reversion frequency of *ts*J12 ($10^{-4}$) is similar to that of other mutants in the series, and this mutant can be rescued by a single restriction fragment in marker rescue experiments. In contrast, *ts*L14 reverts with low frequency ($<10^{-6}$) and cannot be rescued by any one fragment in a series of fragments representing the entire genome (unpublished data).

Mutant *ts*A of strain 17 complemented all 15 *ts* mutants of strain KOS and was assigned to a separate complementation group.

To examine the uniformity of complementation patterns obtained when more than one representative of a group was tested, two additional mutants of the KOS series, *ts*A24 and *ts*F25, were also tested. *ts*A24 and *ts*F25 yielded results identical to those of *ts*A1 and *ts*F18, respectively, in that they complemented the representative mutants of all five complementation groups of strain 17 efficiently (data not shown).

The standard set of HSV-1 *ts* mutants was thus composed of mutants representing 16 complementation groups.

**(ii) Complementation between mutants in the standard set and mutants in other series.** Three series of *ts* mutants have been derived from the KOS strain of HSV-1 by Schaffer et al. (22), Hughes and Munyon (14), and Vonka (unpublished data). The standard set of HSV-1 mutants was next tested against *ts* mutants in the latter two series. The results of these tests are shown in Table 3.

Although *ts*P23 had complemented all mutants of strain 17 (Table 2), it failed to complement four of the mutants in the two other KOS series. Because of its unusual genetic and biological properties which will be discussed below, *ts*P23 was placed in a separate complementation group.

In addition to the multiple negative results obtained with *ts*P23, it is evident that *ts*P23, *ts*7 and *ts*3 did not complement any mutant well. Mutant *ts*3 could be a double or triple mutant (1-3, 1-4, and 1-14); however, the consistently low CI obtained with this mutant makes this conclusion tenuous in the absence of genetic and physical mapping data.

With these exceptions, all but two other mutants fell into groups represented by mutants in the standard set. Two were shown to represent new groups: *ts*478 and *ts*7. Only *ts*661 exhibited an overlapping pattern of complementation not involving *ts*P23. *ts*661 is probably a double mutant because the two mutants that it does not complement are separated by additional cistrons (23). *ts*901 is a single mutant in the *ts*B2 (1-2)

TABLE 2. *Complementation between ts mutants of HSV-1 strains KOS and 17*

| Mutants of strain KOS | Mutants of strain 17 | | | | | Complementation group | Potential members of group |
|---|---|---|---|---|---|---|---|
| | A | D | F | G | I | | |
| A1 | +[a] 1,704[b] | + 315 | + 52 | + 2,311 | + 244 | 1-1 | A1 |
| B2 | + 4,459 | − *1.1* | − 8.8 | + 7,671 | + 18,000 | 1-2 | B2, D |
| C4 | + 3,667 | + 66 | + 756 | + 1,467 | + 1,216 | 1-3 | C4 |
| D9 | + 8,565 | + 9,105 | + 60,067 | + 2,439 | + 2,039 | 1-4 | D9 |
| E6 | + 4,958 | + 626 | + 16,040 | + 189 | + 1,504 | 1-5 | E6 |
| F18 | + 233 | + 97 | + 81 | + 21,100 | + 3,620 | 1-6 | F18 |
| G3 | + 1,771 | + 2,417 | + 126 | − 599 | + 685 | 1-7 | G3 |
| I11 | + 12,000 | + 6,294 | + 26,690 | + 191,000 | + 297 | 1-8 | I11 |
| J12 | + 444 | + 658 | − *1.0* | + 5,374 | − *1.3* | 1-9 | (J12),[c] F, I |
| K13 | + 124 | + 20 | + 29 | + 269 | + 38 | 1-10 | K13 |
| L14 | + 23 | − *0.2* | − *0.7* | + 1,100 | + 181 | 1-11 | (L14),[c] D, F |
| M19 | + 10,364 | + 500 | + 5,763 | + 436,700 | + 3,473 | 1-12 | M19 |
| N20 | ND[d] 576 | ND 423 | ND 706 | ND *0.7* | ND 4,150 | 1-13 | N20, G |
| O22 | + 877 | + 93 | + 219 | + 2.0 | + 1,192 | 1-14 | O22 |
| P23 | ND 2,100 | ND 380 | ND 280 | ND 30 | ND 11 | 1-15 | P23 |
| | A | | | | | 1-16 | A |

[a] + and − are results of qualitative complementation tests. For further explanation, see text.

[b] Numbers are CI obtained in quantitative complementation tests. Pairs with indices of 10 or greater were not retested. Pairs which failed to complement, yielded CI of less than 10, or yielded disparate results in the two types of test were repeated two to five times. In these cases values represent the average of all tests; results of quantitative tests were regarded as definitive. Numbers in italics indicate negative indices.

[c] Potential double mutants are shown in parentheses.

[d] ND, Not done.

cistron. Because *ts*L14 also contains a defect in cistron 1-2, it would not be expected to complement *ts*901.

The results of complementation tests comparing mutants in the standard set with mutants derived from strains HFEM and 13 are shown in Table 4. Again, *ts*P23 yielded negative or low, positive indices with nearly half the mutants tested. Excluding results with *ts*P23, mutants *ts*B1, B7, LB4, LB5, and D10 were shown to constitute new groups, whereas all other mutants were assigned to existing groups. Overlapping patterns of complementation not involving *ts*P23 were evident in the case of *ts*LB2 and LS2. Because *ts*L14 is probably a double mutant, *ts*LB2 was assigned to group 1-2 with *ts*B2. The failure of *ts*LS2 to complement two mutants which lie some distance apart on the KOS linkage map (23) suggests that *ts*LS2 is a double mutant. The mutation in *ts*LB3 is probably in the F cistron of strain 17 (i.e., cistron 1-11), otherwise it would not have complemented *ts*B2.

The overlapping patterns of complementation noted in Tables 2, 3, and 4 suggest that *ts*L14 and *ts*B2 should not have complemented in previous studies (22). By present standards, however, they did complement, although inefficiently (CI = 3.6). Such inefficient complementation in retrospect may have been intracistronic.

**(iii) Complementation tests among *ts* mutants of HSV-1 representing putative new complementation groups.** Eight of the 43 HSV-1 *ts* mutants were found to complement all mutants (except *ts*P23) in the standard set of test mutants and were, therefore, assigned to

TABLE 3. *Complementation between HSV-1 ts mutants in the standard set and other ts mutants of strain KOS*[a]

| Mutants in the standard set | Mutants of strains: | | | | | | | | | Complementation group | Potential members of group |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | KOS 1.1 | | | | | | | KOSpp601 | | | |
| | 84 | 478 | 656 | 661 | 901 | 822 | 833 | 3 | 7 | | |
| A1 | + 27,000 | + 5,294 | − *1.1* | + 9,600 | + 343 | + 17 | + 42,727 | + 39 | + 13 | 1-1 | A1, 656 |
| B2 | + 1,210 | + 3,111 | + 622 | + 653 | − *1.9* | + 30 | + 18,235 | + 25 | − 12 | 1-2 | B2, 901 |
| C4 | − 40 | + 63 | + 34,400 | + *0.8* | + 980 | − 225 | − *0.4* | − *0.6* | + 6.8 | 1-3 | C4, (661), 833, (3) |
| D9 | + *1.9* | + 113 | + 34,800 | + 4.2 | + 564 | − 100 | − 13 | + *0.6* | + 17 | 1-4 | D9, 84, (3) |
| E6 | − 4.0 | + 469 | + 1,520 | + 12 | + 1,494 | + 230 | + 19,166 | + 35 | − 7.3 | 1-5 | E6 |
| F18 | + 46 | + 465 | + 661 | + 2,333 | + 376 | + 72 | + 14,285 | + 32 | + 21 | 1-6 | F18 |
| G3 | + 38,000 | − 28 | + 2,890 | + 424 | + 183 | + 45 | + 4,181 | + 4.2 | + 3.0 | 1-7 | G3 |
| I11 | + 340,000 | + 944 | + 24,400 | + 2,000 | + 565 | + 59 | + 3,181 | + 30 | + 6.8 | 1-8 | I11 |
| J12 | + 13,000 | + 1,062 | + 17,600 | + 1,273 | + 240 | − *0.6* | + 15,000 | + 14 | + 6.4 | 1-9 | (J12), 822 |
| K13 | + 786 | + 344 | + 76,000 | + 154 | + 238 | + 51 | + 1,791 | + 30 | + 4.0 | 1-10 | K13 |
| L14 | + 18 | − 1,000 | + 3,080 | + 35 | − *0.4* | + 2.8 | + 9,523 | + 19 | + 2.8 | 1-11 | (L14), 901 |
| M19 | + 8,000 | + 17,500 | + 144,000 | + 520 | + 518 | + 15 | + 15,384 | + 8.4 | + 14 | 1-12 | M19 |
| N20 | + 682 | + 421 | + 376 | + 169 | + 197 | + 22 | + 9,393 | + 33 | + 21 | 1-13 | N20 |
| O22 | + 21 | + 44 | + 1,289 | + *1.3* | + 352 | + 34 | + 435 | + *1.2* | + 8.2 | 1-14 | O22, (661), (3) |
| P23 | − *0.9* | + 4.6 | + 35 | + 5.1 | + 230 | + 11 | + *1.0* | − *0.02* | − *0.7* | 1-15 | P23 |
| A | + 24,000 | + 1,062 | + 1,880 | + 8,800 | + 1,877 | + 26 | + 7,000 | + 18 | + 7.6 | 1-16 | A |
| | | 478 | | | | | | | | 1-17 | 478 |
| | | | | | | | | | 7 | 1-18 | 7 |

[a] See footnotes to Table 2 for explanation of results.

putative new complementation groups. To confirm the uniqueness of their functions, they were tested among themselves by the quantitative complementation test. The results of these tests are shown in Table 5. Complementation was demonstrated to occur between mutants in all pairwise combinations. Therefore, each of these mutants was shown to represent a new complementation group. Mutant pairs *ts*7 + *ts*B1, *ts*7 + *ts*LB5, *ts*7 + *ts*D10 and *ts*LB4 + *ts*LB5 yielded low, but positive indices. Similar results were obtained in three separate tests of these mutant pairs. The low indices obtained with *ts*7 are consistent with previous findings (Table 3).

Quantitative complementation tests have thus resulted in the identification of 23 complementation groups from among 43 *ts* mutants of HSV-1 (Tables 2 to 5).

**Mixed infections resulting in equivocal and anomalous patterns of quantitative complementation.** CI in positive quantitative tests ranged from 2.0 (e.g., Table 4, *ts*K13 + *ts*LB3) to 440,000 (e.g., Table 4, *ts*M19 + *ts*G5); 72% of positive indices were greater than 50. Of the 123 positive indices with values of less than 50, nearly half were produced in mixed infections with 5 mutants (e.g., *ts*P23, *ts*LB2, *ts*C4 [strain 13], *ts*3, and *ts*7). Negative indices ranged from 0.02 (e.g., Table 3, *ts*P23 + *ts*3) to 1.9 (e.g., Table 3, *ts*D9 + *ts*84).

Although the results of most quantitative tests were unequivocal, certain mutants exhibited equivocal or anomalous behavior in quantitative tests. A careful survey of results indicates that such behavior is a consequence of the properties of individual mutants rather than the properties

TABLE 4. *Complementation between HSV-1 ts mutants in the standard set and mutants derived from strains HFEM and 13*[a]

| Mutants in the standard set | Mutants of strains: HFEM[b] B1 | B5 | B7 | N103 | HFEM[c] LS1 | LB1 | LB2 | LS2 | LB3 | LB4 | LB5 | 13 D10 | C4 | G5 | Complementation group | Potential members of group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | + 1,531 | + 892 | + 87 | − *0.6* | + 110 | + 219 | + 333 | + 157 | + 23 | − 582 | − 279 | + 103 | + 6.0 | + 300,000 | 1-1 | A1, N103 |
| B2 | + 121 | + 340 | + 70 | + 3,333 | + 6,500 | + 11 | − *0.5* | + 371 | + 5.0 | + 7,895 | + 5,333 | + 37 | + 21 | + 18,888 | 1-2 | B2, LB2 |
| C4 | − 20 | + 2,281 | + 85 | + 14,166 | + 45 | + 44 | + 8,666 | + 57 | + 15 | + 4,081 | + 2,842 | + 54 | + 6.3 | + 370,000 | 1-3 | C4 |
| D9 | + 22 | + 545 | + 172 | + 885 | + 14 | + 137 | + 852 | + 153 | + 5.4 | + 354 | + 9,268 | + 352 | + 13 | + 3,210 | 1-4 | D9 |
| E6 | + 2,143 | + 1,438 | + 75 | + 16,800 | + 60 | + 179 | + 3,800 | − *0.5* | + 37 | + 8,077 | + 37 | + 239 | + 11 | + 2,900 | 1-5 | E6, (LS2) |
| F18 | + 2,914 | + 585 | + 280 | + 1,515 | + *0.9* | + 2.5 | + 3.4 | − *0.9* | + 2.2 | + 2,041 | + 36 | + 120 | + 13 | + 450 | 1-6 | F18, LS1, (LS2) |
| G3 | + 239 | + 119 | + 32 | + 9,629 | + 530 | − *1.9* | + 3,400 | + 517 | + 4.1 | + 295 | + 611 | + 117 | − *0.6* | + 120,000 | 1-7 | G3, LB1, C4 |
| I11 | + 1,470 | + 56 | + 142 | + 8,235 | + 55 | + 54 | + 11,600 | + 1,183 | + 14 | + 632 | + 7,222 | + 1,371 | + 14 | + 400,000 | 1-8 | I11 |
| J12 | + 7,818 | + *1.2* | + 138 | + 23,077 | + 250 | + 15 | + 740 | + 63 | + 3.0 | + 7.4 | + 222 | + 104 | + 17 | + *1.8* | 1-9 | (J12), B5, G5 |
| K13 | + 1,214 | + 191 | + 65 | + 6,000 | + 300 | + 400 | + 947 | + 320 | + 2.0 | + 105 | + 1,074 | + 189 | + 10 | + 1,918 | 1-10 | K13 |
| L14 | + 1,090 | + 24 | + 11 | + 1,000 | − 25 | + 4.0 | − *0.2* | + 3.6 | − *0.8* | + 13 | + 12 | + 18 | + 2.2 | + 462 | 1-11 | (L14), LB2, LB3 |
| M19 | + 5,945 | + 241 | + 162 | + 11,677 | + 360 | + 630 | + 21,600 | + 41 | + 33 | + 1,363 | + 14,000 | + 243 | + 13 | + 440,000 | 1-12 | M19 |
| N20 | + 5,416 | + 194 | + 512 | + 30,256 | + 240,000 | + 295 | + 6,956 | + 6.1 | + 13 | + 224 | + 433 | + 217 | + 18 | + 282 | 1-13 | N20 |
| O22 | + 22 | + 36 | + 105 | + 1,194 | + 69 | + 88 | + 1,185 | + 11 | + 16 | + 208 | + 780 | + 250 | + 17 | + 540 | 1-14 | O22 |
| P23 | + *0.9* | + 153 | + *0.5* | + 94 | − 30 | + 32 | + 12 | + 2.7 | + 13 | + 194 | + 3.6 | + *1.9* | + *0.2* | + 48 | 1-15 | P23 |
| A | + 568 | + 2,000 | + 3.7 | + 1,833 | + 50 | + 199 | + 700 | + 30 | + 14 | + 1,522 | + 444 | + 78 | + 9.5 | + 981 | 1-16 | A |

TABLE 4—*continued*

| Mutants in the standard set | Mutants of strains: HFEM[b] B1 | B5 | B7 | N103 | HFEM[c] LS1 | LB1 | LB2 | LS2 | LB3 | LB4 | LB5 | 13 D10 | C4 | G5 | Complementation group | Potential members of group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B1 | | | | | | | | | | | | | | | 1-19 | B1 |
| | | | B7 | | | | | | | | | | | | 1-20 | B7 |
| | | | | | | | | | | LB4 | | | | | 1-21 | LB4 |
| | | | | | | | | | | | LB5 | | | | 1-22 | LB5 |
| | | | | | | | | | | | | D10 | | | 1-23 | D10 |

[a] See footnotes to Table 2.
[b] Mutants of HFEM derived by A. Buchan.
[c] Mutants of HFEM derived by I. Halliburton.

of the test itself.

To illustrate the anomalous behavior of these mutants, the derivation of CI for five representative mutant pairs is shown in Table 6. Data from a typical positive test are illustrated by the pair *ts*I + *ts*B2. In this test, the yield from the mixed infection was clearly greater than the sum of yields in control, single infections. Such efficient complementation is in sharp contrast to the typical negative test illustrated in Table 6 by *ts*N103 + *ts*A1 in which the yield of the mixed infection was slightly less than the sum of the yields from control infections.

Despite the fact that all mutant stocks used in these tests exhibited low levels of leak and reversion in plaque assays, several mutants, notably *ts*3, 7, LB2, and C4 (strain 13), yielded higher levels of leak and/or reversion than other mutants after incubation for 18 h at 39°C which complicated the interpretation of results. As shown in Table 6 (*ts*3 + *ts*G3), the background levels of leak and reversion produced by *ts*3 resulted in a reduction in the CI in this test. Whether the index would have been higher if leak were suppressed by incubation at higher temperature is not known. It was not surprising, therefore, that mutants with the highest levels of leak and reversion yielded consistently lower CI than mutants which did not leak or revert significantly. Consequently, these mutants yielded a greater number of equivocal and negative results.

An additional cause of equivocal and negative results in quantitative tests is illustrated in Table 6 by pairs *ts*P23 + *ts*84 and *ts*P23 + *ts*3. In these tests the yields of mixed infections were significantly lower than the sum of the yields of control infections. In the case of *ts*P23 + *ts*3, this reduction was nearly 1.5 logs. *ts*P23 also suppressed the replication of $ts^+$ revertants of *ts*3 in this test. In contrast to other mutants which consistently yielded low CI, *ts*P23 did not exhibit unusually high levels of leak and/or reversion. These data indicate a strong tendency by *ts*P23 to suppress the production of *ts* virus through complementation mechanisms and $ts^+$ revertants and recombinants. The ability of *ts*P23 to suppress the growth of the wild-type virus by greater than 90% when equal multiplicities of *ts*P23 and wild-type virus are used to infect cells has been noted by J. Jofre (personal communication). In a total of 28 quantitative tests in the present study, *ts*P23 failed to complement eight mutants and produced indices of less than 10 with four other mutants. It is noteworthy that five of eight of the negative quantitative tests were positive in qualitative tests, which involve multiple rounds of replication. Furthermore, the

TABLE 5. *Complementation tests with HSV-1 ts mutants representing putative new complementation groups*

| Mutant | CI from mixed infections with *ts* mutant: | | | | | | |
|---|---|---|---|---|---|---|---|
| | 478 | 7 | B1 | B7 | LB4 | LB5 | D10 |
| 478 | | 16 | 166 | 174 | 1,322 | 4,167 | 253 |
| 7 | | | 5.4 | 64 | 18 | 5.7 | 3.2 |
| B1 | | | | 159 | 34,286 | 13,000 | 1,287 |
| B7 | | | | | 62 | 30 | 47 |
| LB4 | | | | | | 2.6 | 86 |
| LB5 | | | | | | | 210 |
| D10 | | | | | | | |

TABLE 6. *Quantitative complementation tests exhibiting typical positive, negative, equivocal, and anomalous results*

| *ts* Mutants in test | | Virus yield (PFU/ml) | | | | | | CI[a] | Interpretation |
|---|---|---|---|---|---|---|---|---|---|
| | | Mixed infections (A + B) | | Controls | | | | | |
| | | | | A | | B | | | |
| Mutant A | Mutant B | 34°C | 39°C | 34°C | 39°C | 34°C | 39°C | | |
| I | B2 | $1.8 \times 10^5$ | $1.2 \times 10^4$[b] | $<10^1$ | $<10^1$ | $1 \times 10^1$ | $<10^1$ | 18,000[c] | Positive |
| N103 | A1 | $1.1 \times 10^2$ | $<10^1$ | $0.5 \times 10^1$ | $<10^1$ | $1.2 \times 10^2$ | $<10^1$ | 0.9[d] | Negative |
| 3 | G3 | $1.2 \times 10^5$ | $2.7 \times 10^4$[b] | $1.2 \times 10^4$ | $3.3 \times 10^3$[e] | $4.0 \times 10^1$ | $<10^1$ | 9.9[d] | Low positive; leak and reversion by mutant A |
| P23 | 84 | $1.0 \times 10^2$ | $<10^1$ | $4.3 \times 10^2$ | $<10^1$ | $5.0 \times 10^1$ | $<10^1$ | 0.2[d] | Negative; interference by mutant A |
| P23 | 3 | $5.1 \times 10^2$ | $2.3 \times 10^2$[b] | $4.3 \times 10^2$ | $<10^1$ | $1.2 \times 10^4$ | $3.3 \times 10^3$[e] | 0.04[d] | |

[a] $CI = ([A + B]_{39°C})/([A]_{39°C} + [B]_{39°C})$ when infected cells were incubated at 39°C and yields were assayed at 34°C.

[b] Yields of $ts^+$ recombinants and revertants in progeny of mixed infections assayed at 39°C.

[c] This value appears in Table 2.

[d] This table contains data from only one of the two to five tests with these mutant pairs; indices were averaged to produce the results shown in Tables 3 and 4.

[e] Yields of $ts^+$ revertants in progeny of control, single infections assayed at 39°C.

reasons for the efficient complementation observed between *ts*P23 and mutants *ts*A, D, F (Table 2), 901 (Table 3), and B5 and LB4 (Table 4), are unclear.

Of equal significance in the interpretation of the results of quantitative tests is the identification of mutants with overlapping patterns of complementation, i.e., those with multiple *ts* defects as described above.

**Qualitative complementation tests with *ts* mutants of HSV-1.** Although the quantitative test was considered to be definitive in these studies, excellent agreement was observed between the results of qualitative and quantitative complementation tests. With *ts* mutants of strains KOS and 17 (Table 2), no false positive qualitative tests (tests yielding positive qualitative and negative quantitative results) and two false negative tests (tests yielding negative qualitative and positive quantitative results) were observed.

In tests between mutants in the standard set and other *ts* mutants of HSV-1 (Tables 3 and 4), agreement between the two types of tests was also good, albeit less so. Thirteen false negative qualitative results (e.g., Table 3, *ts*C4 [strain KOS] + *ts*84) and 12 false positive results (e.g., Table 3, *ts*D9 + *ts*84) were obtained. Of note is the observation that 5 of the 12 false positive results occurred in mixed infection with *ts*P23. Thus, in a total of 433 qualitative tests, 15 false negative (3.4%) and 12 false positive tests (2.8%) were observed.

**Quantitative complementation tests with *ts* mutants of HSV-2: the standard set of HSV-2 *ts* mutants.** In a collaborative complementation study involving 20 *ts* mutants of HSV-2 strains HGS52 and 186, Timbury et al. (30) identified 18 complementation groups. Representatives of these 18 groups were therefore used in this study as the standard set of HSV-2 *ts* mutants.

**Complementation between the standard set and other HSV-2 *ts* mutants.** The results of complementation tests between *ts* mutants in the standard set and those derived from wild-type strains IPB2, 333, and UW268 are shown in Table 7. The most striking feature of tests with HSV-2 mutants was the fact that CI were consistently 10-fold lower than indices obtained in tests with HSV-1 *ts* mutants. Positive indices ranged from 2.0 to 9,090; 39% were less than 10. In tests which yielded indices between 2 and 5, results were consistent from test to test, i.e., they were always low but positive. Although mutants which exhibited high levels of leak were excluded from the study, HSV-2 mutants consistently yielded higher levels of leak than HSV-1 mutants. Leakiness was thus a significant factor in the generation of low complementation indices with these mutants. Clearly, such inefficient complementation created difficulties in assigning mutants to cistrons.

The failure of *ts*2 to complement 10 mutants also contributed to difficulties in assignment of mutants to cistrons. Failure of *ts*2 to complement was not due to excessive leak. It is possible that *ts*2 may possess similar interfering properties to those of *ts*P23 of HSV-1 described above, i.e., whether *ts*2 is able to suppress replication of wild-type virus at the nonpermissive temperature is not known. To determine the relative distance of *ts*2 from other mutants, all mutants in Table 7 except *ts*1 (strain UW268) were screened for their ability to recombine with *ts*2 at the permissive temperature. Recombination frequencies were additive for the most part and ranged from 3 to 18%, indicating that *ts*2 could recombine efficiently, demonstrating that it was not closely linked to mutants with which it failed to complement.

Of the 11 HSV-2 *ts* mutants tested against the standard set, and excluding the results of tests with *ts*2, all but two, *ts*11 and *ts*12 (strain UW268), failed to complement one or two mutants in the standard set. In mixed infection, *ts*11 + *ts*12 yielded a CI of 35 and were thus shown to be unique. These two mutants were, therefore, placed in new complementation groups 2-19 and 2-20. Four of the 11 mutants against which the standard set was tested exhibited overlapping patterns of complementation (exclusive of tests with *ts*2). Because of this overlap and the overall low indices obtained, these mutants could not be assigned unequivocally to complementation groups. In all, 20 complementation groups were identified by quantitative complementation tests with *ts* mutants of HSV-2.

**Qualitative complementation tests with *ts* mutants of HSV-2.** Of 198 qualitative tests, 9 false negative tests (4.5%) and 12 false positive tests (6.1%) were observed. Eight of the 12 false positive tests occurred with the noncomplementing mutant *ts*2. Thus, although false negative and false positive tests were obtained with both HSV-1 and HSV-2 *ts* mutants, the proportion of anomalous qualitative results was higher in the analysis of HSV-2 *ts* mutants.

**Viral DNA phenotypes of members of complementation groups.** Summaries of the complementation groups and the viral DNA phenotypes of mutants in each group are shown in Tables 8 and 9.

Excluding mutants which exhibited overlapping patterns of complementation, the viral DNA phenotypes of mutants in the 10 HSV-1 complementation groups containing two or more members are in excellent agreement (Table 8). Six of the 23 groups contain $DNA^-$ mutants, 3 contain mutants which synthesize 20% or less of wild-type levels of viral DNA, and 11 contain $DNA^+$ mutants.

Ten of the 20 HSV-2 complementation groups may contain more than one member; 4 of these 10 groups (2-2, 2-3, 2-5, and 2-15) clearly contain two or more mutants. The viral DNA phenotypes of mutants in multimember HSV-2 groups were not in good agreement. The basis for this disparity may lie in the inavailability of definitive complementation data or perhaps to differences in the conditions used for determining viral DNA phenotypes of mutants in different laboratories. In any event, available data clearly indicate that 7 of the 20 groups contain $DNA^-$ mutants (groups 2-1, 2-2, 2-3, 2-4, 2-7, 2-8, and 2-19), 2 contain $DNA^\pm$ mutants (groups 2-13 and 2-14), and 8 contain $DNA^+$ mutants (groups 2-10, 2-11, 2-12, 2-15, 2-16, 2-17, 2-18, 2-20).

Despite the fact that the results of some complementation tests were equivocal and that the viral DNA phenotypes of mutants in certain multimember groups were dissimilar, 6 of 23 HSV-1 groups and 7 of 20 HSV-2 groups were shown to contain $DNA^-$ mutants. Thus, these investigations confirm the previous observation that a large number of cistrons are essential for viral DNA synthesis (21, 27).

## DISCUSSION

Based primarily on the results of quantitative complementation tests, 23 cistrons of HSV-1 and 20 cistrons of HSV-2 have been identified. Although overlapping patterns of complementation complicated the assignment of some mutants to cistrons, they did not diminish the total number of cistrons identified. On the contrary, overlapping patterns of complementation have facilitated the identification of potential double mutants.

The results of quantitative complementation

TABLE 7. *Complementation between HSV-2 ts mutants in the standard set and mutants derived from wild-type strains IPB2, 333, and UW268*[a]

| *ts* Mutants in standard set derived from wild-type strains | | Mutants of strains: | | | | | | | | | | | Complementation group | Potential members of group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IPB2 | | 333 | | UW268 | | | | | | | | |
| HGS52 | 186 | 1 | 42082 | 69 | 74 | 1 | 5 | 6 | 11 | 12 | 19 | 33 | | |
| 1 | | + | + | + | + | + | + | + | + | + | + | + | 2-1 | 1 |
| | | 33 | 85 | 3.0 | 7.0 | 588 | 37 | 5.8 | 24 | 35 | 145 | 83 | | |
| | H9 | − | − | + | + | + | + | + | + | − | − | + | 2-2 | H9, 1, (42082)[b], 19 |
| | | *0.7* | *0.5* | 15 | 3.2 | 18 | 30 | 3.7 | 3.9 | 37 | *1.7* | 55 | | |
| | B5 | + | + | + | + | + | + | − | − | + | + | + | 2-3 | B5, 6 |
| | | 37 | 569 | 70 | 4.2 | 6.7 | 62 | *0.7* | 62 | 338 | 5,790 | 346 | | |
| 9 | | + | − | + | + | + | + | + | + | + | + | + | 2-4 | 9 |
| | | 34 | 48 | 9.0 | 2.5 | 49 | 23 | 16 | 26 | 6.5 | 71 | 3.2 | | |
| | C2 | + | + | + | + | − | + | + | − | + | + | + | 2-5 | C2, 1 |
| | | 2.2 | 171 | 71 | 8.3 | *1.3* | 25 | 3.1 | 60 | 5.7 | 1,212 | 129 | | |
| 11 | | + | + | + | − | + | + | + | + | + | + | + | 2-6 | 11, (74), (5) |
| | | 17 | 5.6 | 4.3 | *1.2* | 4.5 | *0.6* | 2.7 | 4.1 | 5.4 | 23 | 2.6 | | |
| | A8 | + | − | + | − | + | + | + | − | + | + | + | 2-7 | A8, (42082) |
| | | 9.3 | *1.2* | 7.3 | 4.9 | 64 | 23 | 9.3 | 5.5 | 56 | 5.5 | 116 | | |
| 2 | | − | − | + | + | + | + | + | + | + | + | + | 2-8 | 2 |
| | | *0.4* | *1.1* | *0.2* | *0.03* | 20 | *1.0* | *0.03* | *0.6* | *0.03* | *1.8* | *1.6* | | |
| 8 | | + | + | + | + | + | + | + | + | + | + | + | 2-9 | 8, (69), (74) |
| | | 6.3 | 6.0 | *0.8* | *0.5* | 5.2 | 4.5 | 2.8 | 4.2 | 2.9 | 14 | 5.1 | | |
| 3 | | + | + | + | + | + | + | + | + | + | − | + | 2-10 | 3 |
| | | 39 | 12 | 5.3 | 14 | 75 | 48 | 6.6 | 31 | 4.4 | 43 | 22 | | |
| 4 | | + | + | + | + | + | + | + | + | + | + | + | 2-11 | 4 |
| | | 16 | 11 | 3.5 | 2.5 | 72 | 11 | 6.1 | 25 | 9.1 | 430 | 49 | | |
| 5 | | + | + | − | + | + | + | + | + | + | − | + | 2-12 | 5, (69) |
| | | 80 | 5.2 | *0.3* | 4.1 | 28 | 2.5 | 2.1 | 16 | 9.2 | 23 | 8.5 | | |
| 12 | | + | + | + | + | + | + | + | + | + | + | + | 2-13 | 12 |
| | | 9.6 | 4.4 | 7.6 | 4.5 | 39 | 11 | 2.0 | 4.6 | 4.9 | 18 | 8.8 | | |
| 13 | | + | − | + | + | + | + | + | + | + | + | + | 2-14 | 13, (42082) |
| | | 4.6 | *0.7* | 4.7 | 3.1 | 32 | 8.6 | 4.3 | 4.8 | 3.2 | 8.1 | 7.3 | | |
| | D6 | + | + | + | + | + | − | + | + | − | + | + | 2-15 | D6, (5) |
| | | 3.2 | 138 | 67 | 5.9 | 17 | *0.6* | 9.3 | 71 | 8.3 | 1,642 | 326 | | |
| | E7 | + | + | + | + | + | + | + | + | + | + | + | 2-16 | E7 |
| | | 2.2 | 121 | 25 | 6.2 | 37 | 51 | 19 | 12 | 103 | 714 | 6.8 | | |
| | F3 | + | + | + | + | + | + | + | + | + | + | + | 2-17 | F3, 33 |
| | | 13 | 254 | 38 | 8.3 | 236 | 123 | 25 | 29 | 154 | 878 | *1.0* | | |

TABLE 7—*continued*

| *ts* Mutants in standard set derived from wild-type strains | | Mutants of strains: | | | | | | | | | | | Complementation group | Potential members of group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IPB2 | | 333 | | UW268 | | | | | | | | |
| HGS52 | 186 | 1 | 42082 | 69 | 74 | 1 | 5 | 6 | 11 | 12 | 19 | 33 | | |
| G4 | | + | + | + | + | + | + | + | + | + | + | + | 2-18 | G4 |
| | | 19 | 779 | 136 | 7.9 | 350 | 324 | 23 | 366 | 312 | 9,090 | 188 | | |
| | | | | | | | | | 11 | | | | 2-19 | 11 |
| | | | | | | | | | | 12 | | | 2-20 | 12 |

[a] See footnotes to Table 2.

[b] Mutants in parentheses cannot be assigned unequivocally to complementation groups due to overlapping patterns of complementation. These mutants may be double mutants.

tests were considered to be definitive in these studies. A CI of 2 was selected as the value signifying complementation when complementation studies with HSV *ts* mutants were initiated (22). The levels of complementation which represent inter- and intracistronic complementation were not known at that time, and are not known now, due to the absence of fine structural mapping data. Thus, the number of cistrons identified in this study by the quantitative test may be somewhat greater or less than the true number of cistrons represented by the mutants studied. On the other hand, problems also exist with the qualitative test. By using *ts* mutants of HSV-2, the qualitative test yielded results which were similar to those obtained in infectious centers tests (30). Both the qualitative and infectious centers tests involve multiple rounds of replication, and both are complicated by the fact that false positive results could be produced if recombinants can be generated in the absence of complementation. The classical definition of the cistron is based upon virus yields produced during one round of replication (2, 8) and whether recombination can occur in the absence of complementation is not presently known; consequently, the quantitative test was considered to be more definitive.

Because the limits of either test are not presently known, it is essential that the cistron assignments of all mutants be confirmed by recombination and marker rescue tests. Genetic and physical mapping data would be particularly useful in the case of mutants which appeared to be double mutants and those which yielded multiple low and negative results (*ts*P23 of HSV-1 and *ts*2 of HSV-2) in complementation tests. The feasibility of mapping viral genes and polypeptides by analysis of HSV-1 × HSV-2 recombinants has recently been demonstrated (18, 19; N. M. Wilkie, N. D. Stow, H. S. Marsden, V. Brown, R. Cortini, M. C. Timbury, and J. H. Subak-Sharpe, Abstracts of the Third International Symposium On Oncogenesis and Herpesviruses, Cambridge, Mass., 1977). It is anticipated that complementation, recombination and marker rescue tests combined with analyses of HSV-1 × HSV-2 recombinants will ultimately lead to the identification of most essential viral cistrons and to the positioning of these cistrons on the physical maps of HSV DNA.

The identification of additional viral cistrons should continue to constitute a primary goal of genetic studies of HSV. Brown et al. (3) concluded that the HSV genome encodes a minimum of 30 essential functions. Given the large theoretical coding capacity of the genome, this value is probably a considerable underestimate.

TABLE 8. *HSV-1 complementation groups*

| Complementation group | *ts* Mutants derived from strains: KOS | 17 | KOS 1.1 | KOSpp 601 | HFEM[a] | HFEM[b] | 13 | Predominant viral DNA phenotype |
|---|---|---|---|---|---|---|---|---|
| 1-1 | $Al^{-}$[c] | | $656^{-}$ | | $N103^{-}$ | | | − |
| 1-2 | $B2^{-}$ | $D^{-}$ | $901^{-}$ | | | $LB2^{\pm}$ | | − |
| 1-3 | $C4^{-}$ | | $(661^{-})$[d], $833^{-}$ | $(3^{ND})$[e] | | | | − |
| 1-4 | $D9^{-}$ | | $84^{-}$ | $(3^{ND})$ | | | | − |
| 1-5 | $E6^{+}$ | | | | | $(LS2^{+})$ | | + |
| 1-6 | $F18^{+}$ | | | | | $LS1^{+}$, $(LS2^{+})$ | | + |
| 1-7 | $G3^{\pm}$ | | | | | $LB1^{+}$ | $C4^{+}$ | + |
| 1-8 | $I11^{+}$ | | | | | | | + |
| 1-9 | $(J12^{+})$ | $I^{+}$ | $822^{+}$ | | $B5^{+}$ | | $G5^{+}$ | + |
| 1-10 | $K13^{\pm}$ | | | | | | | ± |
| 1-11 | $(L14^{\pm})$ | $F^{+}$ | | | | $LB3^{+}$ | | + |
| 1-12 | $M19^{\pm}$ | | | | | | | ± |
| 1-13 | $N20^{+}$ | $G^{+}$ | | | | | | + |
| 1-14 | $O22^{\pm}$ | | $(661^{-})$ | $(3^{ND})$ | | | | ± |
| 1-15 | $P23^{-}$ | | | | | | | − |
| 1-16 | | $A^{+}$ | | | | | | + |
| 1-17 | | | $478^{+}$ | | | | | + |
| 1-18 | | | | $7^{ND}$ | | | | ND[e] |
| 1-19 | | | | | $B1^{-}$ | | | − |
| 1-20 | | | | | $B7^{-}$ | | | − |
| 1-21 | | | | | | $LB4^{+}$ | | + |
| 1-22 | | | | | | $LB5^{+}$ | | + |
| 1-23 | | | | | | | $D10^{-}$ | − |

[a] Mutants derived from strain HFEM by A. Buchan.
[b] Mutants derived from strain HFEM by I. Halliburton.
[c] +, ±, −, and ND are viral DNA phenotypes from Table 1.
[d] Mutants in parentheses are putative double or triple mutants.
[e] ND, Not done.

TABLE 9. *HSV-2 complementation groups*

| Complementation group | *ts* Mutants derived from strains: KOS | HSG52 | IPB2 | 333 | UW268 | Predominant viral DNA phenotype |
|---|---|---|---|---|---|---|
| 2-1 | | $1^{-}$[a] | | | | − |
| 2-2 | $H9^{-}$ | | 1, (42082)[b] | | $19^{-}$ | − |
| 2-3 | $B5^{-}$ | $6^{-}$ | | | | − |
| 2-4 | | $9^{-}$ | | | | − |
| 2-5 | $C2^{\pm}$ | $10^{-}$ | | | $1^{+}$ | Mixed phenotype |
| 2-6 | | $11^{-}$ | | $(74^{\pm})$ | $(5^{+})$ | Mixed phenotype |
| 2-7 | $A8^{-}$ | | (42082) | | | − |
| 2-8 | | $2^{-}$ | | | | − |
| 2-9 | | $8^{-}$ | | $(69^{+})$ $(74^{\pm})$ | | Mixed phenotype |
| 2-10 | | $3^{+}$ | | | | + |
| 2-11 | | $4^{+}$ | | | | + |
| 2-12 | | $5^{+}$ | | $(69^{+})$ | | + |
| 2-13 | | $12^{\pm}$ | | | | ± |
| 2-14 | | $13^{\pm}$ | (42082) | | | ± |
| 2-15 | $D6^{+}$ | | | | $(5^{+})$ | + |
| 2-16 | $E7^{+}$ | | | | | + |
| 2-17 | $F3^{+}$ | | | | $33^{+}$ | + |
| 2-18 | $G4^{+}$ | | | | | + |
| 2-19 | | | | | $11^{-}$ | − |
| 2-20 | | | | | $12^{+}$ | + |

[a] +, ±, and − are viral DNA phenotypes from Table 1. If phenotype is not given, it has not been determined.
[b] Mutants in parentheses are putative multiple mutants.

The results of the present study demonstrate that most investigators have isolated *ts* mutants in the same genes and that the number of essential cistrons which remain to be identified by techniques already employed is probably small.

An explanation for the failure to identify additional cistrons is readily apparent from Table 1. Of the 72 *ts* mutants tested, 57 were induced with BUdR. Among the 23 HSV-1 complementation groups, 3 contain a single mutant induced by a mutagen other than BUdR (1-10, NTG; 1-12, NTG; and 1-15, AP). Special mention should be made of LS1 and LS2—the only two mutants isolated in the absence of mutagen. Although difficult to isolate, spontaneous mutants are free of mutational artifact. Of the 20 HSV-2 complementation groups, each contains one or more BUdR-induced mutants, reflecting the fact that 26 of the 29 HSV-2 *ts* mutants tested were BUdR-induced.

That mutational hot-spotting may occur with HSV DNA and BUdR must be considered in evaluating the number of cistrons identified in this study. Mutational artifact is a common feature of genetic systems in which mutagens with highly specific modes of action are employed (6, 16). Because nucleotides are not randomly distributed in HSV DNA, large T-rich genes would be preferentially mutagenized by the T-specific mutagen BUdR. In the future it will be of interest to compare the base compositions of the sequences known to encode individual viral genes with the mutagens used to induce mutations in those sequences. For the present, it is clear that further attempts to isolate HSV mutants should utilize mutagens whose specificities differ from that of BUdR. Nitrous acid, which can produce transitions and transversions in all four bases, would seem a prime candidate.

With regard to mutagenesis with NTG, it should be noted that two of the four NTG-induced mutants of strain KOS used to establish the standard set of HSV-1 mutants may be double mutants. Because NTG is notorious for inducing multiple closely linked mutations (10, 26), this mutagen should be used advisedly. In addition to the use of different mutagens, future efforts might also be directed towards the isolation of other types of conditional lethal mutants. Cold-sensitive mutants, for example, have been shown to be clustered in regions of the genome not containing *ts* mutations (24).

The present study was undertaken in part to identify additional essential HSV cistrons from among *ts* mutants of HSV-1 and HSV-2 isolated in different laboratories. New cistrons both of HSV-1 and HSV-2 have, indeed, been identified. However, having demonstrated that most investigators have isolated mutants with defects in the same series of cistrons, the need to identify additional cistrons by new methods is clear. Furthermore, recombination analysis, marker rescue studies, and phenotypic characterization of mutants assigned to the same cistron are essential to verify the results of the complementation tests reported herein.

The second purpose of this study was to facilitate future investigations with HSV *ts* mutants by (i) introducing a uniform system of mutant nomenclature and (ii) identifying mutants in different series which are defective in the same viral genes to avoid duplication of effort in phenotypic characterization of mutants. In addition, it is the intent of the collaborators in this study to make available series of *ts* mutants representing each of the known cistrons to facilitate the identification of additional essential viral genes.

A uniform system of nomenclature for *ts* mutants of HSV-1 and HSV-2 as suggested by Timbury et al. (30) has been introduced. Although the system permits individual laboratories to retain their own mutant designations, it is hoped that publications involving studies with HSV *ts* mutants will include the cistron assignment of mutants by reference to the common system suggested here. Numbers rather than letters have been used to reflect the large coding capacity of the virus, and the prefix "1" or "2" has been used to differentiate HSV-1 and HSV-2 complementation groups, respectively. The proposed system is not intended to be rigid but to form the basis for a system of nomenclature which is bound to evolve as knowledge advances in the field.

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